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Biodegradation of Glycidol and Glycidyl Nitrate†

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When calcium hydroxide is used to desensitize glycerol trinitrate (nitroglycerine)-containing waste streams, the epoxides glycidol and glycidyl nitrate are formed. The epoxide rings of both compounds are unstable to heat in aqueous solutions, and they open to form glycerol 1-mononitrate and presumably glycerol. These transformations were accelerated by microbial activity. Glycerol 1-mononitrate was slowly denitrated to form glycerol. Glycidol and glycidyl nitrate caused base-pair substitutions in the Ames test for mutagenicity, whereas glycerol 1-mononitrate tests were negative.

Wastewater contamination occurs during the manufacture and processing of glycerol trinitrate (GTN), or nitroglycerine. The solubility of GTN in water, its sensitivity to thermal and mechanical shock, and its toxicity preclude direct disposal of GTN-containing waste streams into the environment (9, 10). Microbial systems are effective in biodegrading GTN via successive denitration steps through glycerol dinitrate and glycerol mononitrate isomers, with each succeeding step proceeding at a slower rate (13).

Calcium hydroxide is used to chemically desensitize waste streams containing GTN (11). This treatment causes the disappearance of glycerol tri-, di-, and mononitrate, but the glycidol and glycidyl nitrate which are formed may also present potential pollution problems. Glycidol and glycidyl nitrate contain the highly reactive epoxy group, which tends to confer mutagenic properties (3, 12).

Mammalian studies have demonstrated that glycidol is toxic (5, 7) and alkylates DNA (3). The purpose of this study was to determine the fate of glycidol and glycidyl nitrate when exposed to microbial activity, thus indicating the feasibility of a biological approach to the decomposition of GTN chemical reaction products. An additional objective was to determine the mutagenic properties of both compounds.

MATERIALS AND METHODS

Media. Basal salts media contained 2.0 g of $NH_4H_2PO_4$, 1.0 g of K_2HPO_4, 1.0 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2$, and 0.01 g of NaCl per liter of distilled water adjusted to pH 7.0 before autoclaving. Glucose was added at 1.0 g per liter as indicated. The nutrient broth concentration was 4.0 g per liter. All media containing glycidol and glycidyl nitrate were sterilized through filters of 0.2-μm pore size. Media for continuous cultures were autoclaved, and after cooling, glycidyl nitrate was added through sterile filters. Temperature and light studies were conducted in distilled water at a pH of 6.0. Light studies were conducted under fluorescent lighting with about 10 h of light daily.

Chemicals. Glycidol was purchased from Eastman Kodak Co., Rochester, N.Y. Freshly redistilled samples were used. Glycidyl nitrate was prepared by the method of Ingham and Nichols (4). The identity and purity of the product were confirmed by infrared spectroscopy, nuclear magnetic resonance spectroscopy, and high-performance liquid chromatography (HPLC). Glycerol 1-mononitrate was prepared according to published procedures (9), and its identity and purity were confirmed by melting point, gas chromatography/mass spectroscopy (GC/MS), fourier transform infrared spectroscopy (FTIR), and HPLC.

GC/MS analyses were performed on a Finnigan model 4000 system. Standards (glycerol 1-mononitrate and glycerol 2-mononitrate) and the unknown were chromatographed isothermally at 160°C on a 60-mm glass capillary column coated with SP2100. FTIR analyses were performed on a Nicolet model 7199 with the standard and sample smeared on KBr plates.

Culture conditions. Aerobic batch cultures were incubated in 250-ml Erlenmeyer flasks, each containing 100 ml of medium at 30°C on a New Brunswick G24 environmental incubator shaker. Anaerobic batch cultures were incubated at 37°C in 250-ml Erlenmeyer flasks filled with medium. Throughout this report the designation anaerobic culture conditions refers to the exclusion of aeration. New Brunswick Bio-Flo model C30 bench-top chemostats for continuous cultures were maintained at room temperature under anaerobic conditions with a retention time of 7 days in a 1.4-liter reaction vessel. Incubations were initiated with 100 ppm (100 μg/ml) of glycidol or glycidyl nitrate.

Aerobic cultures were inoculated with activated-sludge microorganisms from the Marlborough Easterly sewage treatment plant (Marlborough, Mass.), and anaerobic cultures were inoculated with microorganisms from digest from the Nut Island sewage treatment plant (Boston, Mass.). The aerobic and anaero-

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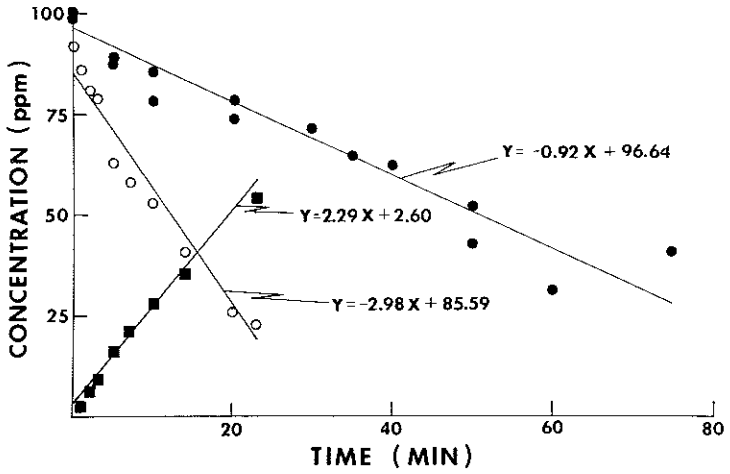


FIG. 1. Effects of a boiling water bath on stability of glycidol (●) and glycidyl nitrate (○) and the formation of glycerol 1-mononitrate (■) from glycidyl nitrate.

bic sludges contained <0.1 and 1.7% total solids. Sludge samples were diluted 100-fold with 0.85% KCl and filtered, and 0.5 ml was added to the culture flasks.

HPLC. Glycidyl nitrate and glycerol 1-mononitrate concentrations were determined on a DuPont 830 liquid chromatograph with a Perkin-Elmer LC55 variable wavelength detector at 195 nm and a Columbia Scientific Supergrator-2 programmable computing integrator. The mobile phase was acetonitrile-water (10/90 [vol/vol]). Culture medium samples were clarified by centrifugation and filtered. Injections of 5 μl were made into a DuPont Zorbax ODS reverse-phase column (25 cm by 4.6 mm) at room temperature and 1,200 lb/in^2. Retention times were about 6 min for glycerol 1-mononitrate and 22 min for glycidyl nitrate. The detection limit was 1 ppm.

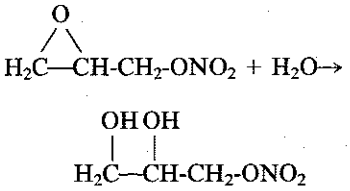
GC. Analysis of glycidol was performed on a Perkin-Elmer model 3920 gas chromatograph equipped with a flame ionization detector and a model 1021A electronic noise filter (Spectrum Scientific Corp.). Nitrogen carrier gas flowed at 30 ml per min through a stainless steel column (46 by 0.32 cm) packed with Poropak Q-S (80–100 mesh). Samples were clarified by centrifugation and filtered, and 3-μl volumes were injected directly on the column. The column temperature was 170°C, and the detector was at 250°C. The detection limit was 0.6 ppm.

Extraction of glycerol 1-mononitrate. A sample of the biologically produced glycerol 1-mononitrate was prepared by continuous extraction of a sample of chemostat effluent for 24 h with ether. The ether was evaporated, and the residue was examined by thin-layer chromatography (TLC), FTIR, and GC/MS. TLC was performed on silica gel plates without fluorescent indicator, developed in benzene-ethanol (95/5), sprayed with diphenylamine in ethanol (5%), and visualized under UV light for 5 to 10 min.

Mutagenicity testing. The Ames screening test for mutagenicity was performed with glycidyl nitrate, glycidol, and glycerol 1-mononitrate according to standard procedures (1, 2). Five strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537, and TA1538) were used to test concentrations from 5 to 5,000 μg per plate with and without metabolic activation.

RESULTS

Distilled water solutions of glycidol and glycidyl nitrate at pH 6.0 were heat sensitive (Fig. 1). In a boiling water bath the half-life of glycidol was 52.5 min and that of glycidyl nitrate was 14.4 min. Thus, under these conditions glycidol required about 3.2 times longer to decompose than glycidyl nitrate. As glycidyl nitrate decomposed, there was a corresponding formation of glycerol 1-mononitrate. The principal reaction appears to be hydrolytic cleavage of the epoxide ring according to equation 1.



(1)

Glycidyl nitrate was not photosensitive (Fig. 2). There was no significant difference in the rate that glycidyl nitrate hydrolyzed in samples kept at room temperature in the light or dark for 30 days. Glycerol 1-mononitrate formed at equivalent rates.

Glycidol disappeared from filter-sterilized solutions at room temperature, but the rate of decomposition was accelerated by microbial activity (Fig. 3). In sterile, aerobic, batch-culture experiments, 36% of the initial 100 ppm of glycidol remained after 17 days. Under sterile

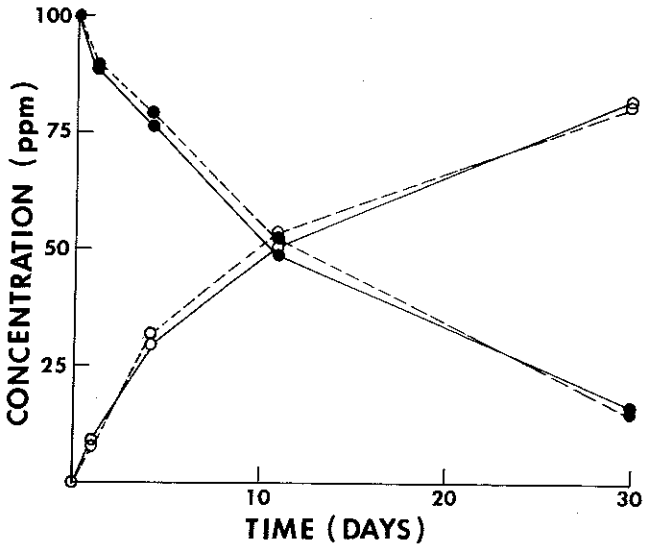


FIG. 2. Photosensitivity of glycidyl nitrate (●) at room temperature in the light (——) and dark (-------) and the formation of glycerol 1-mononitrate (○).

anaerobic conditions 11% of the glycidol remained. Under aerobic conditions in inoculated nutrient broth cultures, glycidol was below the limits of detection after about 4 days. Levels of glycidol (the sole source of carbon present) were below detection limits after about 10 days under both aerobic and anaerobic conditions.

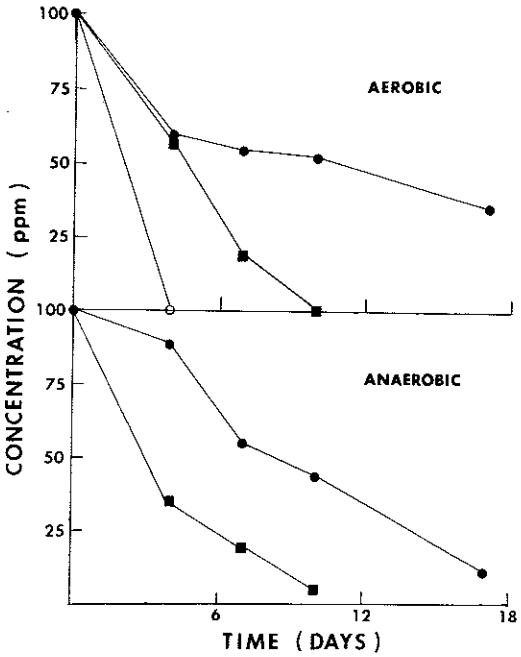


FIG. 3. Decomposition of glycidol in batch cultures under aerobic and anaerobic conditions with filter-sterilized distilled water (●), nutrient broth (○), and basal salts (■) media.

Under batch-culture conditions the epoxide ring of glycidyl nitrate opened even at low temperatures (Fig. 4). At 3°C 47% of the initial 90 ppm of glycidyl nitrate remained after 28 days in sterile nutrient broth flasks. There was no acceleration of decomposition in inoculated flasks at 3°C. Similar results were obtained for sterile control and inoculated flasks with basal salts media with and without glucose. There were no differences in the levels of glycerol 1-mononitrate that formed in sterile and inoculated flasks in the three media.

At 30°C under aerobic conditions (Fig. 4), no detectable glycidyl nitrate remained after 28 days in sterile and 14 days in inoculated nutrient broth flasks. During the same time the glycerol 1-mononitrate level reached 90 ppm in the sterile nutrient broth, whereas in the inoculated broth it peaked at 42 ppm and then gradually declined with time. Data for experiments conducted in different media under the same conditions are not presented in full, but are summarized. In basal salts media with and without glucose, no evidence was found for an accelerated rate of decomposition of glycidyl nitrate due to microbial activity. The concentration of glycerol 1-mononitrate decreased slightly in inoculated flasks with basal salts with and without glucose after about 14 days, whereas in sterile controls no decrease occurred from the 90 to 100 ppm levels reached.

At 37°C under anaerobic conditions (Fig. 4), no detectable glycidyl nitrate remained after 14 days in sterile and 7 days in inoculated nutrient

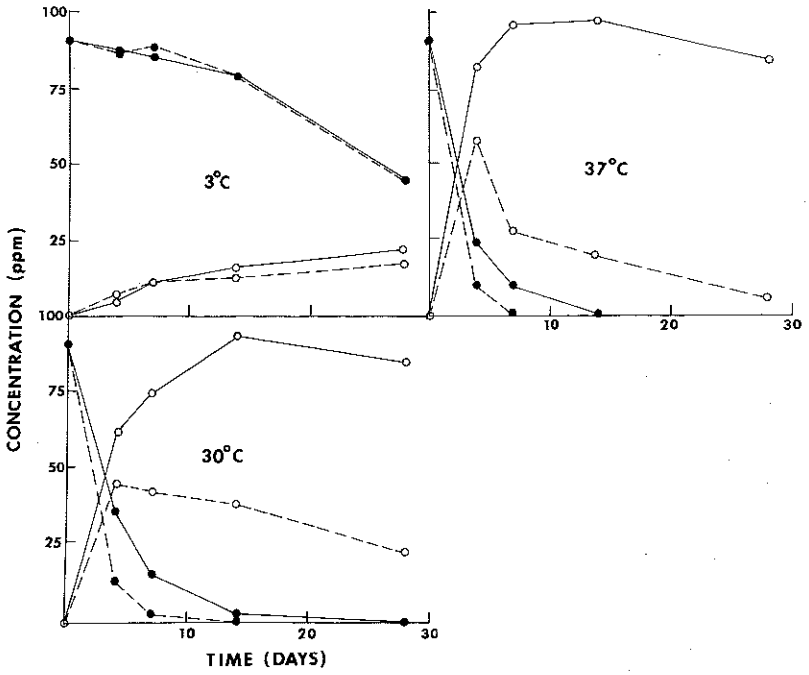


FIG. 4. Decomposition of glycidyl nitrate (●) in batch cultures and the formation of glycerol 1-mononitrate (○) in filter-sterilized nutrient broth (——) and inoculated nutrient broth (------).

broth flasks. The concentration of glycerol 1-mononitrate rose to 90 ppm in the sterile flasks and 58 ppm in inoculated flasks, decreasing to below 10 ppm in the latter. There was no significant difference in the rates of disappearance of glycidyl nitrate and glycerol 1-mononitrate between control and inoculated flasks in basal salts media with or without glucose under anaerobic conditions (data not presented).

Figure 5 illustrates the data from a continuous culture system under anaerobic conditions at room temperature. The glycidyl nitrate concentration of the filter-sterilized medium in the reservoir decreased from 100 to 7 ppm during 22 days. During the same time the concentration of glycerol 1-mononitrate in the reservoir rose to 100 ppm. The decomposition of glycidyl nitrate in the culture vessel that was originally 100 ppm was accelerated by microbial activity; by 6 days less than 5 ppm remained. During this period the level of glycerol 1-mononitrate reached a maximum of 50 ppm in the culture vessel. There was no detectable glycidyl nitrate in the product reservoir, and levels of glycerol 1-mononitrate ranged from 15 to 30 ppm.

Samples from the culture vessel were used for the extraction and analysis of glycerol 1-mononitrate formed in the biological systems. The structure of the product and its identity with the synthesized standard was confirmed by TLC, FTIR, and GC/MS. TLC analysis showed that the extracted glycerol 1-mononitrate had the same R_f as the synthetic standard. The FTIR traces of the sample and standard contained characteristic absorbance bands at 1,050 cm^{-1} ($-CH_2OH$), 1,120 cm^{-1} (-CHOH), and 1,650 cm^{-1} ($-ONO_2$). The GC/MS analysis revealed mass ion peaks at 30, 31, 43, 46, and 61, whereas the relative intensities of the m/z 31 and m/z 61 peaks were the most useful in distinguishing between glycerol 1-mononitrate (m/z 61 > m/z 31) and glycerol 2-mononitrate (m/z 31 > m/z 61). GC retention time and m/z 61 > m/z 31 mass ion intensity confirmed the presence of glycerol 1-mononitrate.

The Ames testing revealed the mutagenic potential of glycidol and glycidyl nitrate (Fig. 6). Both compounds caused significant increases in the rates of back mutation over controls with or without metabolic activation. Significant increases were evident without activation at levels of 50 to 5,000 μg of glycidol per plate with strains TA1535 and TA100, and 500 to 5,000 μg of glycidyl nitrate per plate with TA100 and 50 μg to 5,000 μg per plate with TA1535. Results shown in Fig. 6 are without metabolic activation. With activation the number of revertant colonies increased in most cases, although the lower limits for positive findings remained unchanged. The levels of back mutation are higher for glycidol than for glycidyl nitrate in all cases. None of the other three strains tested showed

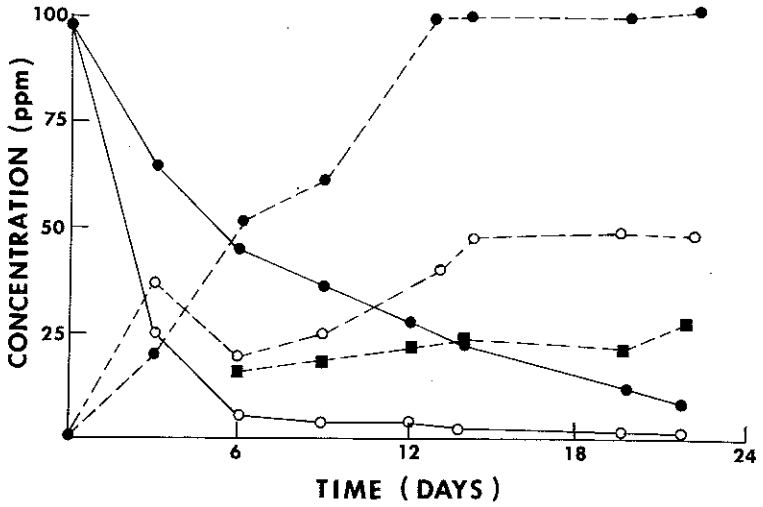


FIG. 5. Disappearance of glycidyl nitrate (——) and the formation of glycerol 1-mononitrate (-------) under continuous culture in the sterile medium (●), culture vessel (○), and product reservoir (■).

significant increases in rates of back mutation. Glycerol 1-mononitrate tested negative with all of the five strains examined.

DISCUSSION

With glycidol present even as the sole carbon source, there was a significant acceleration in the rate of epoxide scission due to microbial activity under both aerobic and anaerobic conditions. Whether this accelerated effect is through primary activity directly on the compound or through secondary effects, such as microbially mediated changes in the media, remains to be shown.

Anaerobic nutrient broth culture samples could not be analyzed for glycidol because the GC method used for analysis was subject to

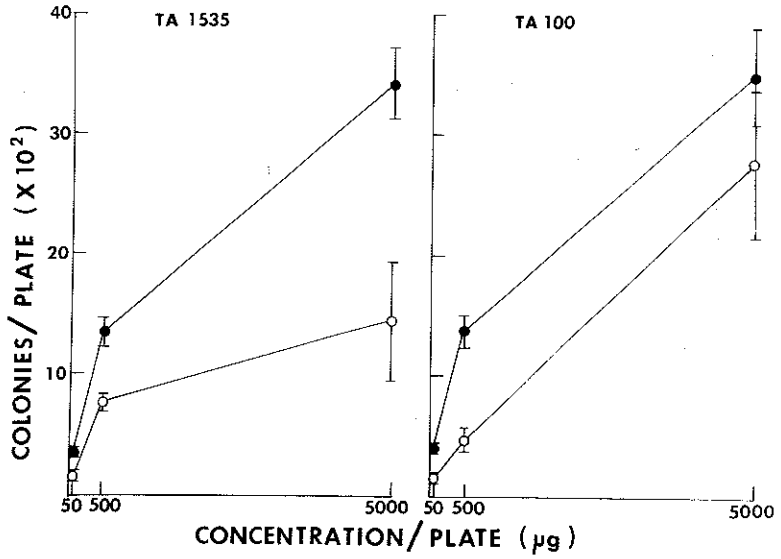


FIG. 6. Ames test results illustrating dose-response curves with glycidol (●) and glycidyl nitrate (○) without metabolic activation. Revertant colonies per plate ± 1 standard deviation. Background controls for TA100 and TA1535 were 133 ± 34 and 24 ± 8 without activation. Not shown are values for 5 μg per plate, which were not significant based on the criterion of a threefold increase in numbers of revertant colonies per plate over background. All values shown are significant except for 50 μg of glycidyl nitrate per plate with TA100.

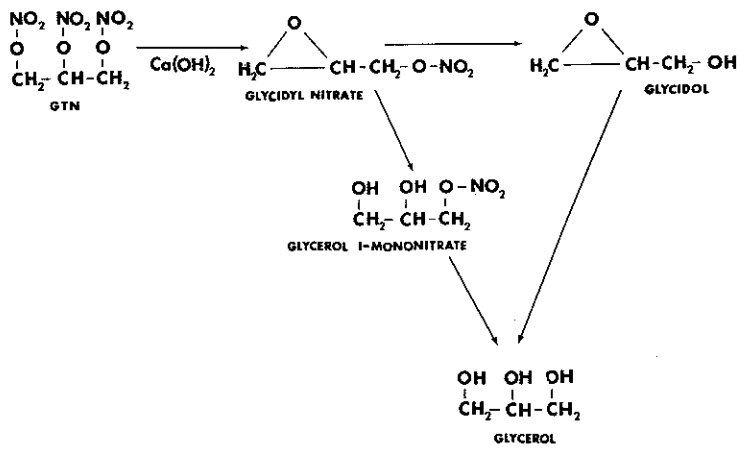


FIG. 7. Scheme for chemical and biological degradation of GTN.

interferences. It would be expected that glycidol would decompose under these conditions since decomposition occurred with glycidol present as the sole carbon source, as well as with added glucose. A spectrophotometric method for the colorimetric determination of glycidol (6) was found unacceptable due to interferences from nutrient broth and alkaline pH's.

In the nutrient broth batch cultures at 30 and 37°C, there was evidence for an accelerated rate of transformation of glycidyl nitrate to glycerol 1-mononitrate due to microbial activity. This increase was greater than that due to chemical hydrolysis alone. This conclusion is supported by the absence of a difference in the rate of transformation of sterile and inoculated media at 3°C where microbial activity is minimized. Even at this temperature glycidyl nitrate underwent some chemical hydrolysis. In continuous culture there was a more pronounced acceleration of the transformation of glycerol 1-mononitrate due to microbial activity than in batch cultures.

Glycerol 1-mononitrate was slowly metabolized in batch and continuous cultures. In continuous culture with a 7-day retention time, influent glycerol 1-mononitrate attained a maximum concentration of about 100 ppm; in the culture vessel and spent media it was considerably reduced. Presumably with longer retention times or more nearly optimal conditions, complete disappearance of this ester could be achieved. Glycerol 1-mononitrate is probably transformed by microbial activity to glycerol through a denitration step, as are other nitrate esters (11, 13). No evidence was found for other nitrate esters by TLC with the diphenylamine spray reagent or by HPLC at 195 and 254 nm, wavelengths at which other nitrate esters could have been detected. ^{14}C-labeled glycerol was found in urine from rats treated with [1,3-^{14}C]glycerol 1-mononitrate (9). Glycerol is readily assimilated by bacteria.

The pathway for microbial metabolism of GTN chemical transformation products is shown in Fig. 7. No attempt was made to identify glycerol in any effluent. The transformation from glycidyl nitrate to glycerol 1-mononitrate to glycerol proceeds more slowly with each succeeding step. The steps from glycidol to glycerol and from glycidyl nitrate to glycerol 1-mononitrate occur spontaneously in aqueous solutions above freezing, but are accelerated through microbial activity.

Glycidol and glycidyl nitrate gave positive results in the Ames test, producing base-pair substitutions, whereas the transformation product glycerol 1-mononitrate tested negative. Our results corroborate those of other investigators who have examined glycidol in the Ames test with an incomplete battery of *Salmonella* strains (8, 12). As with GTN, glycerol 1-mononitrate produces toxicity in mammals (9).

A waste stream containing GTN would be contaminated with epoxides upon chemical treatment with calcium hydroxide. Biological treatment of these epoxides may be a feasible approach to alleviating this pollution problem. Both glycidol and glycidyl nitrate, the principal products, are mutagens and potential environmental hazards. These contaminants disappear during biological treatment through cleavage of the epoxide rings, and mutagenic activity is lost. Glycerol 1-mononitrate, although toxic, is biodegraded. A biological treatment process could be developed to accelerate the transformation rates under less energy-rich media conditions.

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